The Use of Glucose Oxidase (Notatin) for the Determination of Glucose in Biological Material and for the Study of Glucose-producing Systems by Manometric Methods

By D. KEILIN AND E. F. HARTREE, *Molteno Institute, University of Cambridge*

(Received 7 July 1947)

Glucose oxidase or notatin, obtained in a highly purified state from culture media of *Penicillium notatum* (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, 1945), catalyzes the aerobic oxidation of glucose to gluconic acid. The fact that this enzyme shows both a high affinity and a marked specificity for glucose (see preceding paper) suggested the possibility of its utilization for the estimation of glucose in presence of other sugars or of proteins, and for the study of the kinetics of different enzyme reactions which involve the liberation of glucose from its derivatives such as di- and polysaccharides, phosphorylated sugars and glucosides. Manometric methods, owing to their rapidity and simplicity, have great advantages over other methods of analysis on a micro scale and have been applied, for example, to the estimation of urea by urease, glutathione by glyoxalase, succinic acid by muscle preparations and amino-acids by specific bacterial decarboxylases. An outstanding merit of such methods is that they can be made far more specific than any purely chemical methods. The use of notatin extends the application of manometric methods to all reactions which involve the liberation of glucose.

In this paper we shall describe various examples of the use of notatin for the manometric estimation of glucose, and for manometric studies of the kinetics of reactions catalyzed by different enzymes.

EXPERIMENTAL

If the amount of glucose present in a solution is being determined, the notatin must be added from dangling tubes when the manometers have been equilibrated in the thermostat. On the other hand, in studying the rate of production of glucose by a hydrolytic enzyme either this enzyme or its substrate (e.g. a glucoside) should be added from the dangling tube in order that glucose production may be followed in terms of O_2 uptake from the beginning of the reaction. It is important in all experiments of the latter type that the notatin should be present in excess, and that the rate of production of glucose should be the limiting factor in the overall reaction. Under such conditions there will be no accumulation

of glucose in the reaction mixture and the rate of O_2 uptake catalyzed by notatin will be a genuine measure of glucose production, i.e. of the activity of the hydrolytic enzyme.

The oxidation of glucose by the highly purified glucose oxidase proceeds quantitatively and the O_2 uptake under specified conditions can be taken as a measure of the glucose present (Keilin & Hartree, 1945*a*). The primary oxidation reaction is the formation of gluconic acid and H_2O_2 :

When using very pure glucose oxidase (notatin) in slightly acid solution (the optimum pH is 5.6) the H_2O_2 is not decomposed at an appreciable rate, and the final value of the O_2 uptake will be 1 mol. O_2 /mol. glucose (124.4 μ l. O_2 /mg. glucose). As a general rule part of the H_2O_2 decomposes, and in order to estimate glucose by a manometric technique it is essential to add catalase which will decompose the H_2O_2 as it is formed. The nett reaction is then

and the O_2 uptake will be 62.2 μ l./mg. glucose. We have shown previously that the addition of certain alcohols to an enzyme system producing H_2O_2 will, in presence of catalase, lead to an oxidation of the alcohol to the aldehyde (Keilin & Hartree, 1936*a*, 1945*a*). This coupled oxidation of the alcohol is catalyzed by catalase. The overall reaction is now

and, if the alcohol is present in excess, the O_2 uptake again reaches the value of 124.4 μ l./mg. glucose.

It is important in all quantitative estimations of O_2 uptake to ascertain that under the experimental conditions the H_2O_2 formed by reduction of O_2 is either quantitatively decomposed into water and O_2 or completely utilized in a coupled oxidation. Under either of these conditions there is a stoichiometric relationship between the glucose oxidized and the O_2 uptake of the system. In absence of any substances which can undergo coupled oxidation in presence of H_2O_2 and catalase the former will be quantitatively decomposed into water and O_2 , but

in practice part of the H_2O_2 will as a rule be utilized in coupled oxidations. It is therefore advisable in all cases to investigate the effect on the system of ethanol, the presence of which will ensure that any H_2O_2 not utilized for other coupled oxidations will be utilized for oxidation of ethanol to aldehyde.

Whereas in our experiments we have made use of pure glucose oxidase, satisfactory results can be obtained with crude material and even with culture media of *P. notatum* obtained as described by Coulthard *et al.* (1945). Such enzyme preparations will naturally be more liable to exhibit coupled oxidations than the pure material.

Detergents and proteolytic enzymes are sometimes used for the preparation of tissue extracts suitable for analysis, and experiments were carried out in order to determine their effect upon notatin. We have found that after 1 hr. incubation at 39° and pH 5.6 with 0.2% sodium deoxycholate, 0.2% sodium dodecylsulphate, 1% pepsin or 1% papain (in presence of cysteine) the catalytic activity of notatin was unimpaired.

Oxygen uptakes were measured in Barcroft differential manometers, usually at 39° . The flasks contained a total volume of 3.3 ml., one of the components of the system being added from a dangling tube so that the complete O_2 uptake from zero time could be recorded. Whenever possible the pH of the reaction mixture was at or near 5.6, although any pH between 3.5 and 7 is permissible. The highly purified sample of notatin (see preceding paper) was made up as a 0.1% aqueous solution which was stable for 1 week at 5° . Catalase was obtained from horse liver by the method previously described (Keilin & Hartree, 1945b).

Estimation of glucose in blood plasma

Samples of oxalated blood of a rat (A) or of cats (B, C, D) were centrifuged immediately after removal from the animal and each sample was tested in two manometers. The right-hand flask of one manometer received 2 ml. plasma, 0.1 ml. catalase and buffer (0.25 M-phosphate, pH 5.6) to 3.0 ml. while the second manometer received the same ingredients plus 0.1 ml. 20% (v/v) ethanol. In each flask was placed a dangling tube containing 0.3 ml. notatin solution (300 μ g.). Both left-hand flasks received 3.3 ml. buffer. After equilibration in a thermostat at 39° the dangling tubes were dislodged and the O_2 uptakes followed manometrically. In presence of ethanol the H_2O_2 formed during the oxidation of glucose should be quan-

titatively utilized for oxidation of ethanol according to equation (3). In absence of ethanol the reaction should follow equation (2) unless there are in plasma oxidizable substances which can take the place of ethanol in coupled oxidation.

Fig. 1 shows the results obtained with plasma A. In absence of ethanol, 1 mg. glucose $\equiv$ 62.2 μ l. O_2 and the final O_2 uptake corresponds to 3.63 mg. glucose. In presence of ethanol 1 mg. glucose $\equiv$ 124.4 μ l. O_2 and the O_2 uptake corresponds to

Fig. 1. Oxidation by notatin of the glucose in 2 ml. rat plasma in presence and in absence of 20 mg. ethanol. Each right-hand flask contains 300 μ g. notatin, 0.1 ml. catalase, 2 ml. plasma and 0.1 M-phosphate buffer pH 5.6. Temp. = 39° .

3.37 mg. glucose. In all cases the experiments without ethanol gave O_2 uptakes slightly more than half that observed in presence of ethanol. In Table 1 are summarized the results obtained with the 4 samples of plasma together with figures for reducing sugar estimated by the Hagedorn & Jensen method (1923). The latter method gives results which are

Table 1. Estimation of glucose in blood plasma (a) manometrically with notatin + catalase $\pm$ ethanol (see Fig. 1 for details) and (b) by the Hagedorn & Jensen method

	Glucose found by manometric methods (mg.)		Hagedorn & Jensen estimation of glucose (mg.)		
	- Ethanol	+ Ethanol	Total reducing substances as glucose	Non-fermentable as glucose	Glucose (corrected)
2 ml. plasma A (rat)	3.63	3.37	4.05	—	—
2 ml. plasma B (cat)	4.40	4.06	4.73	—	—
2 ml. plasma C (cat)	3.20	2.72	3.45	0.63	2.82
2 ml. plasma D (cat)	3.53	2.99	3.67	0.66	3.01

higher than those obtained by either manometric method. However, in two cases a correction was made for the non-fermentable reducing substances in the plasma by estimating the apparent glucose content before and after fermenting the glucose by means of yeast. The procedure described by Mann (1946) was used for this purpose. When this correction was applied the agreement between the Hagedorn & Jensen method and the manometric method in presence of ethanol was very satisfactory.

Estimation of glucose in other biological media

The method just described for blood plasma can be applied generally to plant and animal fluids or tissue extracts. In the case of some plant extracts (e.g. potato) it is necessary to destroy the phenol-oxidases which, in presence of the natural substrates in the extracts, give rise to a rapid O_2 uptake with consequent formation of highly coloured oxidation products. The enzyme is rapidly destroyed by heating to 80° for 10–15 min., and after such treatment the O_2 uptake of the extract alone will be very small and can be applied as a correction to the uptake

By the manometric method as little as 0.1–0.2 mg. glucose may be accurately estimated. The main advantages of the method are its simplicity, the fact that it is not necessary to remove proteins and, above all, the high specificity of notatin for glucose. On the other hand the method requires about 10 times the quantity of material necessary for the Hagedorn & Jensen method. Furthermore, the manometric method cannot be used for analysis of whole blood as part of the oxyhaemoglobin will become oxidized to methaemoglobin with consequent liberation of the bound O_2 . When the quantity of glucose is too small for manometric estimation notatin may be utilized in connexion with the Hagedorn & Jensen method. Thus by estimation of reducing substances by the latter method, before and after incubation with notatin, the glucose may be accurately estimated. This was done by Mann (1946) who has shown that the reducing values of seminal plasma before and after treatment with glucose oxidase were approximately the same. In this way he demonstrated the virtual absence of glucose in the seminal plasma, and confirmed his discovery that the main reducing carbohydrate of seminal plasma is D-fructose.

Table 2. *Glucose estimation in various plant extracts using notatin*

(300 μ g. notatin, 0.1 ml. catalase, 0.1 M-phosphate buffer pH 5.6. Temp. = 39° . O_2 uptakes shown are the final steady values.)

System in manometer	Plant juice alone	Plant extract + notatin + catalase*		Plant extract + notatin + catalase + ethanol†		Plant extract + notatin + catalase + ethanol + invertase†	
	O_2 uptake (μ l.)	O_2 uptake (μ l.)	Glucose (mg.)	O_2 uptake (μ l.)	Glucose (mg.)	O_2 uptake (μ l.)	Sucrose (mg.)
2 ml. cabbage sap	0	286	4.60	495	3.97	—	—
1 ml. potato press juice (heated)	7	173	2.64	308	2.42	—	—
0.5 ml. beetroot press juice	3	179	2.83	323	2.57	538	3.28
0.5 ml. mangold press juice	0	245	3.94	391	3.14	1048	10.05

* 1 mg. glucose $\equiv$ 62.2 μ l. O_2 (see p. 230).

† 1 mg. glucose $\equiv$ 124.4 μ l. O_2 (see p. 230).

in presence of notatin. Tests have been carried out on cabbage sap and on press juices from potato, beetroot and mangold, and in each case the extract was tested (a) with notatin and catalase and (b) with notatin, catalase and ethanol, at 39° under the above conditions. The results are summarized in Table 2. As in the case of plasma it is seen that higher values are obtained in absence of ethanol owing to a partial utilization of H_2O_2 for coupled oxidation. The results in presence of ethanol are therefore the more reliable. By carrying out an estimation in presence of invertase, both glucose and sucrose may be determined, the difference in O_2 uptakes in presence and in absence of invertase, being a measure of the glucose equivalent of the sucrose. In such experiments the O_2 uptake reaches a steady value in 20–30 min.

Studies on invertase

(a) *Activity of invertase in vitro.* The standard methods for the study of invertase involve the use of the polarimeter or the periodic acid estimation of reducing sugar. The former gives a continuous record, but cannot be applied to solutions which are cloudy or which contain large quantities of other optically active substances. The latter method only gives the quantity of sucrose inverted at one instant during the reaction, and becomes laborious when continuous records of a number of experiments are required. Neither of these difficulties arise when the reaction is carried out in presence of notatin and the glucose production followed by the O_2 uptake in a manometer. As a source of enzyme British Drug Houses

'invertase concentrate' was used at a dilution of 1 in 250 and the manometric method was checked by estimation of reducing sugar at the end of the experiment.

Two Barcroft manometers were filled as follows: The right-hand flask of each manometer received 0.3 ml. diluted invertase, 0.6 ml. 0.1% notatin, 0.1 ml. catalase, 2.0 ml. 0.25M-phosphate pH 5.6 and 0.3 ml. 10% sucrose in a dangling tube. The left-hand flasks received 3.3 ml. buffer. The experiments were carried out at 20°, and the sucrose was added after equilibration and closing the manometer taps. One manometer was shaken for 28 min. from the addition of sucrose when the O_2 uptake was 312 μ l. At 29 min. 1 ml. liquid from the right-hand flask was added to a hot mixture of 25 ml. 0.45% $ZnSO_4 \cdot 7H_2O$ and 5 ml. 0.1N-NaOH, heated at 100° for 3 min. and filtered. The reducing sugar was estimated in the filtrate by the Hagedorn & Jensen method. The second manometer showed an O_2 uptake of 470 μ l. at 42 min. At 43 min. the reducing sugar in the left-hand flask was similarly estimated. The O_2 uptakes for 29 and 43 min. were calculated from the observed values at 28 and 42 min. respectively, and the results obtained by the two methods are shown in Table 3 where the glucose found manometrically is seen to be very close to the residual reducing sugar (fructose).

Table 3. Invertase activity measured (a) manometrically with notatin, and (b) by estimation of reducing sugar

(0.3 ml. invertase (B.D.H. concentrate 1 : 250), 600 μ g. notatin, 0.1 ml. catalase, 30 mg. sucrose. 0.15M-phosphate pH 5.6. Temp. = 20°.)

	In 29 min.	In 43 min.
$\{O_2$ uptake (μ l.)	312	470
$\{Glucose$ equivalent (mg.)	5.01	7.57
Residual reducing sugar = fructose (mg.) by Hagedorn & Jensen method	5.13	7.40

In Fig. 2 is shown the effect of varying concentrations of sucrose on the activity of an invertase solution at 17° and pH 5.6. Nelson & Sottery (1924) have shown that low concentrations of glucose activate, and higher concentrations inhibit the initial velocity of hydrolysis of sucrose by honey-bee salivary invertase. The rate of inversion was, on the other hand, only slightly affected by fructose. The effect of high concentrations of fructose or invertase could not be readily determined by the usual methods, but by means of notatin it could be shown manometrically that 0.167M-fructose inhibits the rate of hydrolysis of 0.007M-sucrose in presence of invertase by only 18%. The study of the kinetics of the invertase reaction by means of notatin is simplified owing to the complete elimination of glucose which may either accelerate or retard the reaction.

(b) *Activity of invertase in vivo.* Yeasts contain an exceptionally powerful invertase which, from our preliminary experiments, appeared to be far more

active than would be necessary to maintain a sufficient supply of monosaccharide for normal metabolic requirements. It therefore seemed probable that in presence of sucrose, respiring yeast cells would discharge large quantities of invert sugar into the surrounding medium. The yeast selected for this

Fig. 2. Inversion of varying concentrations of sucrose by invertase followed manometrically by oxidation of the liberated glucose by notatin. Each right-hand flask contains 0.2 ml. invertase concentrate (1 : 250), 500 μ g. notatin, 0.1 ml. catalase, 0.2M-phosphate buffer pH 5.6. Figures express concentration of sucrose. Temp. = 17°.

work was *Torulopsis utilis* (National Collection of Type Cultures no. 2576). This yeast does not ferment sugars under aerobic conditions, and it therefore becomes a simple matter to compare its metabolic requirements with the activity of the intracellular invertase by a simple manometric technique using notatin.

The organism was grown in Roux flasks on beer wort containing 3% agar and adjusted to pH 6.5. The flasks containing the medium were sterilized for 20 min. at 100° on three successive days. After inoculation the flasks were incubated at 30° for 2 days and then at room temperature for 3 days. The cells were collected in distilled water and washed repeatedly with distilled water in centrifuge tubes. A suspension in water was prepared containing 0.2 mg. dry wt./ml. Table 4 shows the ingredients of the right-hand flasks of the four manometers used for the test. The left-hand flasks received 3.0 ml. buffer and a roll of filter paper soaked in 10% NaOH.

In view of the very high invertase activity as little as 0.1 mg. dry weight of the yeast is sufficient. The Q_{O_2} was estimated separately on a larger quantity of yeast (Fig. 3). After closing the taps the manometers were shaken for 15 min. prior to addition of sucrose from the dangling tubes: during this period the O_2 uptake was negligible. At

15 min. the sucrose was added and the O_2 uptake followed for the next 55 min. (Fig. 3). At this point the reaction was stopped by addition to each right-hand flask of a hot mixture of 1 ml. 4.5% $ZnSO_4 \cdot 7H_2O$, 0.2 ml. $N-NaOH$ and 6 ml. H_2O . The quantities of reducing sugars, estimated as already described, in the protein-free filtrates, are given in Table 4 together with the corresponding O_2 uptakes. The residual reducing sugar in manometer 1 = invert sugar formed minus sugar oxidized by yeast, and this value must be corrected by the figure from manometer 4 which represents the reducing value of the yeast. Similarly the reducing sugar formed in manometer 2 represents sugar inverted minus sugar oxidized by yeast and notatin, and this figure must be similarly corrected from manometer 3.

Table 4. *Invertase activity of Torulopsis utilis determined (a) manometrically with notatin, and (b) by estimation of reducing sugars in presence and in absence of notatin*

(The volumes in the right-hand flasks of the manometers were made up to 3 ml. with phosphate buffer (0.25 M, pH 5.6) and the central tube received 0.3 ml. 10% $NaOH$ and a roll of filter paper. The left-hand flasks received 3 ml. buffer and a roll of filter paper soaked in 10% $NaOH$.)

Components of system (right-hand flask)	Manometer no.			
	1	2	3	4
0.5 ml. <i>T. utilis</i> (0.1 mg.)	+	+	+	+
300 μ g. notatin + 0.1 ml. catalase	-	+	+	-
0.3 ml. 10% sucrose in dangling tube	+	+	-	-
O_2 uptake in 55 min. (μ l.)	9	250	0	0
Total reducing sugar at 55 min. (mg.)	8.63	4.83	0.02	0.02

Hence glucose oxidized = $(1 - 4) - (2 - 3) = 3.80$ mg. From the O_2 uptake in manometer 2 the glucose content is $250/62.2 = 4.01$ mg. A correction must be applied for the slow oxidation of inverted sucrose (9 μ l. O_2) detected in manometer 1. The complete oxidation of 1 mol. invert sugar by yeast requires 12 atoms oxygen instead of the 1 atom utilized by notatin. The correction to be applied to the O_2 uptake in manometer 2 is therefore $9/x$ where x may be as high as 12. The correction is within experimental error, and the glucose oxidized according to the manometric method may be taken as 4.00 mg. This value may be too high for two reasons: (a) The sucrose used in these experiments showed a slight reducing action in the Hagedorn-Jensen estimation (= 0.06 mg./manometer) and part at least of this impurity may be glucose, (b) a slight coupled oxidation of substances in the yeast or the sucrose may take place (as in the case of the estimation of glucose in plasma (Table 1)). The agreement between the two methods is therefore satisfactory.

From the above results it is seen that 0.1 mg. *T. utilis* produces 2×3.8 mg. invert sugar in 55 min., i.e. 1 mg. produces 83 mg./hr. The complete oxidation of this quantity of sugar by the organism would require $83 \times 62.2 \times 12 = 62,000 \mu$ l. O_2 . In other words if the yeast could oxidize the monosaccharides to CO_2 and H_2O as rapidly as they were produced

its Q_{O_2} would be 62,000 instead of 112, the figure obtained experimentally (Fig. 3). There is thus a considerable 'overflow' of invert sugar.

Fig. 3. Inversion of sucrose by yeast (*Torulopsis utilis*) followed manometrically in terms of oxidation of the liberated glucose by notatin. Experiment carried out at 39° in four manometers (1-4, see Table 4). Each manometer receives 0.1 mg. (dry wt.) *T. utilis* in 0.1 M-phosphate buffer pH 5.6; manometers 2 and 3 also contain 300 μ g. notatin and 0.1 ml. catalase. 0.3 ml. 10% sucrose was added after 15 min. to manometers 1 and 2. Manometer 5: O_2 uptake by 4.24 mg. yeast in presence of 0.3 ml. 10% sucrose. The central tubes of all flasks receive 0.3 ml. 10% $NaOH$ and a roll of filter paper. The numbering of the curves corresponds to that of the manometers.

Experiments similar to those just described have been carried out on baker's yeast, brewer's yeast, *B. subtilis* and mycelia of *P. notatum* (see preceding paper). The first 3 organisms showed a similar large 'overflow' of invert sugar, but *P. notatum* was unable to invert sucrose.

Maltase

The study of maltase activity by standard methods is straightforward since only 2 sugars are present in the reaction mixture, viz. maltose and glucose. Nevertheless the activity of the enzyme is most conveniently studied with the help of notatin. As a source of maltase we have used a Lebedew juice prepared by dispersing 50 g. air-dried brewer's yeast in 150 ml. water, incubating 3 hr. at 39° and filtering. In presence of catalase and 300 μ g. notatin, 0.5 ml. Lebedew juice showed, under the experimental conditions already described for invertase, a very slow O_2 uptake (40 μ l./hr.). On addition of maltose O_2 uptakes up to 1500 μ l./hr. were observed. It was of interest to find that the maltase activity of living brewer's yeast was insufficient to provide any 'overflow' of glucose which could be detected by

means of notatin when the yeast was shaken in a manometer with maltose. The invertase activity of the brewer's yeast, however, was such as to provide a very large 'overflow' (see above).

Amylases

These hydrolyzing enzymes may be divided into two classes: the α -amylases present in pancreas, saliva, micro-organisms and malt, which break down starch into dextrins of mol. wt. 1000–3000 and finally into maltose and glucose, and the β -amylases of ungerminated seed grains which yield dextrins and β -maltose but no glucose (Hopkins, 1946). The formation of glucose and maltose from starch by

Fig. 4. Oxidation by notatin of the glucose produced from starch and glycogen by saliva and by salivary amylase. Temp. = 39°. All manometers contain 300 μ g. notatin, 0.1 ml. catalase and 50 mg. polysaccharide in 0.1 M-phosphate pH 5.6. Manometers 1 and 2 contain 0.5 ml. saliva; manometers 3 and 4 contain 6 mg. amylase. The numbering of the curves corresponds to that of the manometers.

α -amylases has been studied by Somogyi (1938, 1940) and by Myrbäck (1941, 1942). While the latter has found evidence that malt amylase produces the two sugars simultaneously, the former considers that maltose precedes glucose in the reaction catalyzed by α -amylases from animal sources, and that the final quantities of maltose and glucose produced correspond to about 60 and 10 % respectively of the total starch. Such investigations are hampered by the complexity of the mixtures of breakdown products and by difficulties in the determination of glucose and maltose in mixtures. The preferential fermentation of glucose by baker's yeast at pH 8.4, though far from satisfactory, has until now been the only method of determining glucose in presence of maltose. The value of notatin in such investigations is obvious and we have been able to demonstrate manometrically the formation of

glucose not only from soluble starch, but also from glycogen using both saliva and an α -amylase isolated from saliva by the method of Hanes & Cattle (1938). The manometer flasks contained 300 μ g. notatin, 0.1 ml. catalase, 50 mg. carbohydrate and a suitable amount of amylase. The final concentration of phosphate buffer (pH 5.6) was 0.1 M and the experiments were carried out at 39°. The O_2 uptakes are plotted in Fig. 4 where curves 1 and 2 show the formation of glucose in presence of 0.5 ml. saliva from glycogen and soluble starch respectively. Curves 3 and 4 represent corresponding experiments with 6 mg. amylase. In both cases there was no O_2 uptake in presence of maltase after a correction had been applied for the slow rate of oxidation of maltose (see preceding paper). Furthermore the polysaccharides themselves were shown to be free from glucose. These experiments offer proof of the accepted view that glucose arises from the splitting of α -1, 4 linkages in the polysaccharides and not from hydrolysis of maltose. At the same time they demonstrate a method for the study of the kinetics of glucose production by amylases; a problem to which there has hitherto been no satisfactory experimental approach. The similarity in the course of the reactions with the two polysaccharides is very striking.

Hydrolysis of glucose phosphates

The phosphatases which hydrolyze glucose-1-phosphate (Cori ester) and glucose-6-phosphate (Robison ester) are widely distributed in animal tissue. A convenient source of enzyme is the intestinal mucosa and a crude preparation was obtained as follows: the small intestine of a rabbit was well irrigated with tap water, the mucosa scraped off and ground in a mortar with an equal volume of acetate buffer (0.1 M, pH 5.6) and sand. The suspension was decanted from the sand after standing 5 min. The standard method of studying the enzyme hydrolysis of these esters is to determine at intervals the increase in inorganic phosphorus. On addition of notatin to the system, however, a continuous manometric record of glucose production can readily be obtained.

For the study of the Robison ester three manometers were filled as shown in Table 5, the notatin being dissolved in 0.3 ml. water and placed in a dangling tube. After equilibration and closing the taps, the notatin was added. The course of the reaction is shown in Fig. 5 (full lines). At 30 min. the manometer readings were taken, giving the O_2 uptakes shown in Table 5, and the reactions stopped by the addition of 3 ml. 20 % trichloroacetic acid to each right-hand flask. After standing 1 hr. the mixtures were filtered and the filtrate and washings made up to 25 ml. Inorganic P was estimated by the Fiske-Subbarow method (Rona, 1929). The

increase in inorganic P due to phosphatase action is the P content of manometer 1 less that of 2 and 3, i.e. $778 - 32 - 154 = 592 \mu\text{g. P}$. The O_2 equivalent of the liberated P is similarly, from Table 5, $221 - 13 - 0 = 208 \mu\text{l.}$ which corresponds $31 \times 208 / 11.2 = 577 \mu\text{g. P}$. The figures obtained by the two methods are in good agreement.

Table 5. *Hydrolysis of Robison ester (glucose-6-phosphate) by a suspension of rabbit intestinal mucosa*

(Volumes made up to 3.3 ml. with 0.2M-acetate buffer pH 5.6.)

Components of system (right-hand flask)	Manometer no.		
	1	2	3
1 ml. mucosa	+	-	+
300 $\mu\text{g.}$ notatin + 0.1 ml. catalase	+	+	-
1 ml. Robison ester (0.1M)	+	+	-
O_2 uptake in 30 min. ($\mu\text{l.}$)	221	0	13
Inorganic P at 30 min. ($\mu\text{g.}$)	778	32	154

Fig. 5. Rates of hydrolysis at 39° of Robison ester (—) and of Cori ester (---) catalyzed by a phosphatase preparation from intestinal mucosa. Glucose production followed by means of O_2 uptakes in presence of notatin. 0.1M-acetate buffer pH 5.6. Curves 1 and 4: phosphatase, notatin, catalase and ester. Curves 2 and 5: notatin, catalase and ester. Curves 3 and 6: phosphatase only. For quantities see Tables 5 and 6.

A parallel investigation of the hydrolysis of Cori ester gave the results summarized in Table 6 and Fig. 5 (dashed lines). The hydrolysis of this ester is slower and the sample contained a considerable quantity of inorganic P. The results obtained after allowing the reactions to proceed for 85 min. are as follows: increase in inorganic P = $1298 - 363 - 493 = 442 \mu\text{g. P}$. Glucose liberated $\equiv 240 - 39 - 35 = 166 \mu\text{l.}$ $\text{O}_2 \equiv 460 \mu\text{g. P}$. In view of the large blanks the agreement is most satisfactory.

Table 6. *Hydrolysis of Cori ester (glucose-1-phosphate) by a suspension of rabbit intestinal mucosa*

(Volumes made up to 3.3 ml. with 0.2M-acetate buffer pH 5.6. Temp. = 39° .)

Components of system	Manometer no.		
	1	2	3
1 ml. mucosa	+	-	+
300 $\mu\text{g.}$ notatin + 1 ml. catalase	+	+	-
1 ml. Cori ester (0.1M)	+	+	-
O_2 uptake in 85 min. ($\mu\text{l.}$)	240	39	35
Inorganic P at 85 min. ($\mu\text{g.}$)	1298	363	493

The initial very rapid O_2 uptakes in manometer 1 in each set of experiments is due to the accumulation of glucose during the interval required for equilibration. A more exact study of the kinetics of the reactions would require that either the phosphatase preparation or the ester be added from the dangling tube (see Introduction). The control experiment phosphatase + notatin indicated that the former contained no appreciable amount of glucose.

Activity of glucosidases

(1) *Emulsin* catalyzes the hydrolysis of amygdalin or mandelonitrile gentiobioside, the reaction proceeding as follows:

The first stage is usually determined in a polarimeter, but is more readily followed with the use of notatin in the manner already described. The HCN produced in the second stage will, however, inactivate the catalase which is an essential component of the notatin system if a quantitative measure of glucose production is required. The most convenient way to overcome this difficulty is to add methaemoglobin which has a high affinity for HCN and which forms a stable CN-compound (Keilin & Hartree, 1936b) so protecting the catalase from inhibition. By this method curves 1 and 2 of Fig. 6 were obtained with 5 and 2.5 mg. emulsin respectively at 22° and pH 5.6. Each right-hand manometer flask received 2 ml. 10 % crystalline methaemoglobin solution (for preparation see Keilin & Hartree, 1935) in addition to emulsin, amygdalin, notatin and catalase, and after taking readings for 45 min. the reaction mixtures were examined with a microspectroscope. At acid pH methaemoglobin shows four absorption bands in the visible region, the most prominent band being in the red at $632 \text{ m}\mu$., while its CN' derivative shows one broad band in the green. In both cases it was readily seen that only part of the methaemoglobin was combined with HCN. One molecule of methaemoglobin (mol. wt. 68,000) contains 4 Fe atoms and

will combine with 4 molecules of HCN. The glucose produced in Exp. 1 (Fig. 6) can be determined from the O_2 uptake, and if it is assumed that the two phases of hydrolysis of amygdalin take place simultaneously then 2 molecules of glucose will appear for each molecule of HCN. On these grounds it can be calculated that if the O_2 uptake is 400 μ l., the HCN produced will be sufficient to combine with 300 mg. methaemoglobin, i.e. 3 ml. 10 % solution. Since in

Fig. 6. (a) Hydrolysis of amygdalin by emulsin followed in terms of the oxidation of liberated glucose by notatin. Curve 1: 5 mg. emulsin, 60 mg. amygdalin, 2 ml. 10 % methaemoglobin, 300 μ g. notatin, 0.1 ml. catalase, 0.1 M-acetate buffer. Temp. = 22°. Curve 2: as 1 with 2.5 mg. emulsin. (b) Hydrolysis of sinigrin by sinigrinase. Glucose production followed by O_2 uptake in presence of notatin. Curve 3: 1 ml. sinigrin, 1 ml. sinigrinase, 0.1 ml. catalase, 300 μ g. notatin, 0.1 M-phosphate buffer pH 5.6. Temp. = 22°. Curve 4: as 3 without notatin. Curve 5: as 3 without sinigrinase.

Exp. 1 the HCN liberated is less than that required to react with 2 ml. 10 % solution, it follows that the second phase of the hydrolysis is slower than the first. The second phase can best be followed by the microspectroscopic technique already described by us using a double-wedge trough, and periodically matching the absorption spectrum of the reaction mixture with that of optical mixtures of methaemoglobin, and its CN' derivative contained separately in the two compartments of the double-wedge trough (Keilin & Hartree, 1946).

(2) *Emulsin* also catalyzes the hydrolysis of salicin to saligenin and glucose, and the reaction can be followed in the same way although here the methaemoglobin can be omitted.

(3) *Sinigrinase* was characterized by Neuberg & Wagner (1926) as the enzyme hydrolyzing sinigrin, a glucoside present in horse-radish root and mustard seed, the reaction proceeding as follows:

The usual manometric technique with notatin is well suited to the study of the kinetics of this system. Crude sinigrin was prepared by boiling 100 g. crushed white mustard seed with 300 ml. H_2O for 15 min. and filtering with the aid of Hyflo Supercel (Johns-Manville, London). The clear, deep-yellow filtrate was not further purified. Sinigrinase was obtained by the method of Neuberg & Wagner from the mustard seed and its reaction with sinigrin in presence of notatin is shown in Fig. 6 (curves 3, 4, 5).

SUMMARY

1. Glucose oxidase or notatin which catalyzes the aerobic oxidation of glucose to gluconic acid, shows a marked specificity and a high affinity for glucose.

2. The stoichiometric relationship between the glucose oxidized and the oxygen utilized makes possible an accurate manometric estimation of glucose in biological fluids containing other carbohydrates and proteins.

3. The kinetics of different enzyme reactions which yield glucose can be very easily studied manometrically by means of notatin. In this way a continuous record of the hydrolytic process can be obtained in terms of O_2 uptake.

4. The use of notatin has been applied to the manometric study of the reactions catalyzed by invertase *in vitro* and *in vivo*, maltase, salivary amylase, phosphatase, emulsin and sinigrinase.

5. The use of notatin provides a method for the quantitative study of the liberation of glucose from starch and glycogen by α -amylase. It has made possible the direct identification of glucose in such reactions.

6. By means of notatin it can be determined manometrically that living yeast cells (*T. utilis*) invert sucrose 500 times faster than is necessary to provide the monosaccharides necessary for normal respiratory requirements.

REFERENCES

- Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1942). *Nature, Lond.*, **150**, 634.
- Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1945). *Biochem. J.* **39**, 24.
- Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
- Hanes, C. S. & Cattle, N. (1938). *Proc. Roy. Soc. B*, **125**, 387.
- Hopkins, H. H. (1946). *Advances in Enzymology*, **6**, 389.
- Keilin, D. & Hartree, E. F. (1935). *Proc. Roy. Soc. B*, **117**, 1.
- Keilin, D. & Hartree, E. F. (1936a). *Proc. Roy. Soc. B*, **119**, 141.
- Keilin, D. & Hartree, E. F. (1936b). *Proc. Roy. Soc. B*, **119**, 114.
- Keilin, D. & Hartree, E. F. (1945a). *Biochem. J.* **39**, 293.
- Keilin, D. & Hartree, E. F. (1945b). *Biochem. J.* **39**, 148.
- Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 210.
- Mann, T. (1946). *Biochem. J.* **40**, 481.
- Myrbäck, K. (1941). *Biochem. Z.* **307**, 142.
- Myrbäck, K. & Thorsell, W. (1942). *Svensk kem. Tidskr.* **54**, 50.
- Nelson, J. M. & Sottery, C. T. (1924). *J. biol. Chem.* **62**, 139.
- Neuberg, C. & Wagner, J. (1926). *Biochem. Z.* **174**, 457.
- Rona, P. (1929). *Praktikum der physiologischen Chemie*, **2**. Berlin: Springer.
- Somogyi, M. (1938). *J. biol. Chem.* **124**, 179.
- Somogyi, M. (1940). *J. biol. Chem.* **134**, 301.

Filter-paper Partition Chromatography of Sugars

1. GENERAL DESCRIPTION AND APPLICATION TO THE QUALITATIVE ANALYSIS OF SUGARS IN APPLE JUICE, EGG WHITE AND FOETAL BLOOD OF SHEEP

BY S. M. PARTRIDGE, WITH A NOTE BY R. G. WESTALL

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge

(Received 9 June 1947)

The development by Consden, Gordon & Martin (1944) of a partition method of chromatography, in which sheets or strips of filter paper are used instead of the familiar packed column, has already shown its usefulness as a method for the qualitative analysis of protein or peptide hydrolysates for amino-acids, especially in circumstances where the amount of material available is small. Although the method is not suitable for obtaining accurate quantitative results, it has the special advantage that use can be made of very small differences in partition coefficient, with the result that useful separations may be obtained with as many as nineteen or twenty amino-acids on the same chromatogram. The present communication describes an extension of the method to the qualitative analysis of sugars, either in biological fluids or in the hydrolysis products of polysaccharides. Preliminary results have already appeared (Partridge, 1946).

The essentials of the method are as follows: A small quantity of the solution to be analyzed is introduced in the form of a circular spot near the top of a sheet or strip of filter paper. The paper is then hung vertically from a trough containing a solvent which has previously been saturated with water, so that the top edge of the paper is immersed

in the solvent. The trough and the filter-paper strips are then suspended inside a closed vessel, the atmosphere in which is kept in a condition of saturation both with respect to water and to the solvent. A sharp horizontal liquid front forms, which advances down the paper at a speed dependent upon the physical properties of the solvent used. To irrigate a paper strip some 35 cm. long, 18–24 hr. are usually sufficient, after which the strip may be dried and the new positions of the components of the solution rendered visible by spraying the paper with an appropriate reagent.

Under these conditions, partition of the solute takes place between water bound by the cellulose and the solvent which is moving over the surface of the cellulose fibres. The distance moved by any solute in the direction of flow is then a function of (1) the partition coefficient of the solute between water and the wet solvent, (2) the volume of water bound by unit area of the filter paper, and (3) the volume of wet solvent held by unit area of the filter paper after irrigation. In any single experiment, factors (2) and (3) are substantially constant provided the filter paper is uniform in texture, thickness and water content, so that under ideal conditions the relative distance (R_F) moved by each